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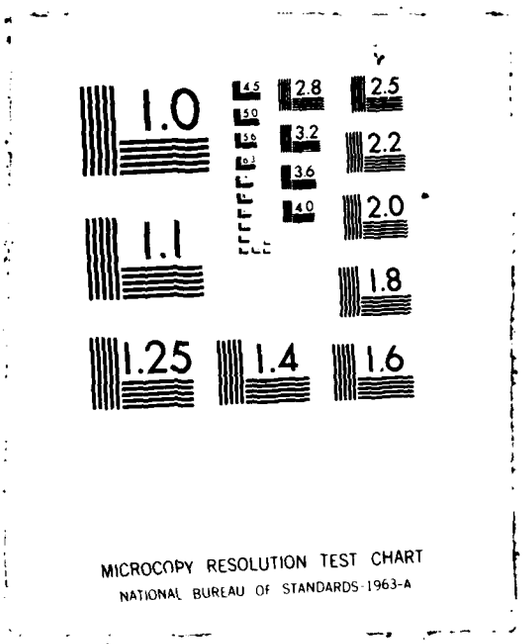
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CARBOXYALKYLATED HEMOGLOBIN AS A POTENTIAL BLOOD SUBSTITUTE

Annual Report

James M. Manning

January 24, 1987

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Carboxymethylated hemoglobin A has been prepared for possible use as a potential blood substitute. The covalently attached carboxymethyl groups on the terminal amino groups of hemoglobin lead to a significantly lowered oxygen affinity. The allosteric effector CO_2^- is also known to lead to a lower oxygen affinity. The stable carboxymethyl derivative is considered to be an analog of the adduct of CO_2^- and hemoglobin. The conclusion is supported by X-ray diffraction analysis. Deoxy hemoglobin A is carboxymethylated more selectively than oxy hemoglobin A. The Bohr effect for the derivative $\alpha_2^{Cm}\beta_2$ is reduced about 25% but the full Bohr effect is retained for $\alpha_2^{Cm}\beta_2$ species. Studies on the cross-linking of the derivative by glycolaldehyde have been initiated. In preliminary studies, the cross-linked carboxymethyl hemoglobin was found to have an oxygen affinity lower than that of unmodified hemoglobin but higher than that of the carboxymethylated derivative. Further studies on the characterization of the cross-linked species are in progress.

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INTRODUCTION

Statement of the Problem Under Study - Our objective is to obtain a useful blood substitute with normal adult HbA as the starting material. For such a derivative to be useful it should have a low oxygen affinity so that oxygen can be readily released to the tissues. To this end we have been studying the carboxymethylation of the N-terminal amino groups of HbA. The initial studies on this derivative showed that it had a low oxygen affinity, a desirable property for a blood substitute (1).

The second objective of the studies is to cross-link this carboxymethylated Hb so that it will be retained longer in the circulation and yet maintain a significant degree of Hb function. For this purpose we are investigating the use of glycolaldehyde, which is a small cross-linking agent of latent reactivity.

Background and Review of Literature - There have been several reports of HbA-based blood substitutes in the literature (2). The major objective of such studies has been to attain an oxygen affinity of the Hb derivative which is compatible with an efficient oxygen delivery to the tissues. Many studies have been done with the pyridoxal phosphate derivative with Hb. This rationale has been based upon the finding that pyridoxal phosphate is a covalent analogue of the natural allosteric modulator, 2,3-DPG. It has been known for many years that under physiological conditions within the red cell the release of oxygen from Hb is facilitated by DPG and also by protons and CO_2. The studies with pyridoxal phosphate have indeed showed that the oxygen affinity of the pyridoxylated derivative is shifted in the desirable direction. The major sites of interaction of pyridoxal phosphate on Hb have been reported to be Val-1(β) and Lys-82(β) (3, 4). However, it could also react with other lysine residues and the extent to which this latter finding

will affect the final homogeneity of the product is not clear at the present time. Nevertheless, this pyridoxylated derivative has shown some very useful properties and it can be considered as a prototype of a covalent modification of Hb whose properties approach those of the desired derivative.

Retention of a Hb-based derivative in the circulation is a second and equally important consideration. There have been a number of reports on the cross-linking of pyridoxylated Hb with glutaraldehyde. In some cases such cross-linking has led to a left shift in the oxygen equilibrium curve that is considerably more than would be desirable. Another problem with glutaraldehyde is the fact that it is not a particularly stable reagent and it is prone to undergo self-condensation in solution. In addition, it is extremely reactive and thus control of products may be difficult to attain.

In our studies, we have used an alternative cross-linking agent, glycolaldehyde, which is a latent cross-linking reagent and intrinsically milder than glutaraldehyde. We have previously examined the cross-linking of the protein ribonuclease with glycolaldehyde and we have shown that it is only the ε-amino groups of lysine residues which are cross-linked by glycolaldehyde.

Our studies on carboxymethylated Hb as another approach at covalent modification of HbA at selective sites were initiated a few years ago based on our interest of mimicing the effect of CO_2 on Hb. It is well known that the natural allosteric modulator CO_2 also leads to a shift in the oxygen equilibrium curve so that a lower oxygen affinity is attained. It was our objective to limit the carboxymethylation so that the reaction took place at selective and limited number of sites, preferably the N-terminal amino groups of both chains. The reaction conditions and identification of the sites of reaction were worked out to achieve these goals (1).

Later studies in another laboratory used the same carboxymethyl derivative to attain a low oxygen affinity of Hb and it was proposed that such a derivative might be a useful blood substitute (5, 6). In those studies, the cross-linking of the Hb derivative was achieved with a dextran-based derivative. Some success was achieved in retaining a low oxygen affinity of the resultant derivative. In addition, it was pointed out by those investigators that this derivative might be a useful one to study the electrostatic properties of the Hb molecule (6). Our rationale in considering this derivative as a potential blood substitute is described in more detail in the next section.

Rationale Used in Current Study - As described above, CO_2 is one of the naturally-occurring allosteric modulators of Hb that lowers its oxygen affinity within the red cell so that oxygen is released more readily to the tissues. Studies on the derivative of CO_2 with Hb ($HbNHCOO^-$)are difficult to perform because the product is labile and difficult to isolate. Hence, we concluded that the stable carboxymethyl derivative ($HbNHCH_2COO^-$) would be easier to study. The rationale for using this derivative also involves the fact that it has been known for many years that small inorganic anions such as chloride can bind to HbA and also lead to a decreased oxygen affinity (7). The mechanism of this effect is not generally fully agreed upon since some investigators consider that the binding of salts to Hb could be a non-specific one. On the other hand, more recent studies from our laboratory and from other laboratories (8-11) have shown that the binding of chloride is specific for certain sites on Hb. One of these sites is the N-terminus of the α-chain and the second site is in the cleft between the two β-chains which usually binds 2,3-DPG in deoxy Hb. However, it was clearly futile to expect that a useful derivative of Hb could be obtained simply by treatment with small non-

covalent inorganic anion such as chloride since these could be readily diffuse from the protein solution. Therefore, the concept of preparing a covalent adduct of Hb which would bind to or near these sites and would mimic the effect of chloride is one that we considered. The attraction to the carboxymethyl derivative is that in addition to the fact that it might mimic some of the effects of CO_2 on Hb, it was also to be considered as a small covalent anion anchored firmly to certain sites on Hb. This proposed analogy has, in general been borne out by these studies we have performed during the past year and which we are continuing to perform. The studies to date including some of those which formed the basis of the study prior to the inception of this contract are described in this report.

The rationale of using glycolaldehyde as a cross-linking agent is described above. The goal is to obtain a cross-linked Hb derivative which has retained some or all of the functional properties of Hb. The actual degree of cross-linking in terms of a desired molecular weight is not currently known. Therefore, we have studied a variety of experimental conditions of cross-linking so that we might obtain either predominantly 64,000 molecular weight species with internal cross-links, or 128,000 or higher molecular weight species of Hb tetramers linked together. These species can be separated and studies on each class are planned.

EXPERIMENTAL METHODS

Preparation of Standards - In order to assess quantitatively the extent of carboxymethylation of the amino groups of the N-terminal valine residues and the ε-NH_2 groups of lysine residues of Hb, the desired derivatives were synthesized, separated by anion exchange chromatography, and characterized. α-N-monocarboxymethylvaline was prepared by incubation of L-valine and a

mixture of 0.25 μCi sodium ^{14}C-glyoxylate (Amersham Company, specific radioactivity 7.3 mCi/mmol) with ^{12}C-glyoxylate (Sigma) and recrystallized sodium cyanoborohydride (Sigma) in a molar ratio of 1:1:10 in 50 mM HEPES buffer, pH 7.0 for 12 hrs at 37°C in a volume 500 μl. N,N-α-dicarboxymethylvaline was synthesized in a similar fashion but the reagents were in a molar ratio of 1:10:100. In this latter preparation, 0.60 μCi of ^{14}C-glyoxylate was used. These two samples were acidified with HCl and lyophilized. N-ε-Monocarboxymethyllysine was prepared by incubation of α-t-butyloxycarbonyl (Boc)-lysine, ^{14}C/^{12}C-glyoxylate (described above), and sodium cyanoborohydride in a molar ratio of 1:1:10 in 50 mM HEPES, pH 7.0 for 4 hrs at 37°C in a volume of 500 μl. The sample was then evaporated to dryness and the t-butyloxycarboyl group was removed by addition of 200 μl 50% trifluoroacetic acid in methylene chloride. After 30 min at room temperature this solution was evaporated to dryness in a Savant microconcentrator.

Each of these compounds was dissolved in 1 M acetic acid. After separate application to a column of Dowex-2 (Bio-Rad, OH-form, 0.9 x 22 cm), each sample was eluted with 1 M acetic acid, 3 M acetic acid, and finally 1 M HCl. This system was a modification of the procedure of Dreze et al (12) originally described for desalting amino acids. Portions of each fraction (800 μl) were mixed with scintillation fluid and the radioactivity was measured in an LKB scintillation counter. The fractions that contained radioactivity were pooled and lyophilized. The two valine derivatives were subjected to elemental analysis. The results, shown below, were consistent with the theoretical values calculated for N-α-monocarboxymethylvaline and N,N-dicarboxymethylvaline.

N-Monocarboxymethylvaline				N,N-Dicarboxymethylvaline			
N	C	H		N	C	H	
8.00	47.99	7.48	theory	5.20	40.30	5.20	theory
7.52	46.56	7.34	found	4.90	39.26	5.10	found

After repeated washing of each sample with D_2O and evaporation in a Savant microconcentrator, ^{1}H-NMR spectra were performed on a Nicolet 300 MHz spectrometer. The spectra confirmed the identities of the valine derivatives as the monocarboxymethyl and dicarboxymethyl derivatives, respectively. These derivatives are ninhydrin-negative. The lysine derivative, which was eluted in 1 M acetic acid, was applied to an amino acid analyzer since it is ninhydrin-positive. Its position coincided with that of a commercial sample of N-ε-monocarboxymethyllysine (Sigma).

Isolation of HbA - Whole blood from normal individuals was centrifuged at 2,000 x g for 10 min and 4°C and the supernatant plama was discarded. After the cells had been washed three times with cold isotonic saline, they were lysed by the addition of an equal volume of distilled water. This solution was then frozen and slowly thawed. The lysate thus obtained was centrifuged at 12,000 x g for 20 min to remove membrane and particulate matter. The lysates were then dialyzed at 4°C against 0.1 M NaCl for removal of organic phosphates. For some studies, the Hb was saturated with CO at all steps of the preparation. For studies with deoxy Hb, CO was not used until after the initial carboxymethylation reaction. Hb concentrations were determined by measurement of the absorbance at either 420 nm or 540 nm.

Reductive Carboxymethylation of HbA - Dialyzed lysates containing HbA (1.5 mM) were treated with a 10-fold molar excess of sodium glyoxylate and a 20-fold molar excess of sodium cyanoborohydride at 25°C in 50 mM potassium phosphate, pH 7.4 for 60 min. When necessary, the concentration of glyoxylate

was determined as the 2,4-dinitrophenylhydrazone derivative. All other chemicals were reagent grade and of the highest purity available. For experiments with liganded Hb, the CO form was used. For studies with deoxy Hb, a specially constructed two-armed Erlenmeyer flask was used for the deoxygenation and the subsequent mixing of reactants (13). After the carboxymethylation reaction, the Hb was saturated with CO and the excess starting materials were removed from the protein by gel filtration on a Sephadex G-25 column (2 x 25 cm) with 50 mM Tris acetate buffer, pH 8.3, as the eluent.

Separation of α- and β-Chains from Carboxymethylated Hb and Preparation of Specifically Modified Hybrids - Pooled fractions representing derivatives Hb_1 and Hb_2 that were isolated by chromatography on DE-52 (Fig. 1) were dialyzed against CO-saturated 0.1 M NaCl. Each tetrameric species was separated into its α- and β-chains by a modification of the method of Njikam et al (14). In this chromatographic system, the negatively charged carboxymethyl groups permit the separation of the modified α-chain from the unmodified α-chain. However, there is no such separation of the carboxymethylated β-chain since both modified and unmodified chains are eluted in the void volume of the column. Since there is more unmodified β-chain in Hb_1 than in Hb_2, we chose to use the more fully carboxymethylated derivative Hb_2 for preparation of hybrid tetramers of Hb modified specifically at the terminus of either the α- or β-chains or at both termini. For preparation of these hybrids, carboxymethylated α- or β-chains were mixed either together to generate $\alpha_2{}^{Cm}\beta_2{}^{Cm}$ or with an equivalent amount of the unmodified β- or α-chain to yield $\alpha_2{}^{Cm}\beta_2$ and $\alpha_2\beta_2{}^{Cm}$, respectively. A 300-fold excess of mercaptoethanol (relative to the concentration of derivatized (SH groups) was added, and, after gentle mixing, the solution was allowed to stand overnight

at 4°C. In this procedure, which is identical with that used for the preparation of specifically carbamylated Hb derivatives (13), the solutions of the Hb chains are used at the pH and ionic strength at which they are eluted from the Cm-52 column (14). The excess 2-mercaptoethanol is removed by dialysis against several volumes of CO-saturated 10 mM NaCl.

Purification of Specifically Carboxymethylated Hb Hybrids - In order to ensure that the hybrid Hb derivatives were free from minor Hb contaminants, each hybrid tetramer was chromatographed on CM-52 (0.9 x 30 cm) after concentration to a volume less than 10 ml in an Amicon cell fitted with a YM-10 membrane. The gradient used for elution of the derivative from the column was from 10 mM potassium phosphate, pH 5.85, 1 mM in EDTA, to 15 mM potassium phosphate, pH 7.9, 1 mM in EDTA (150 ml of each). The buffer was saturated with CO. An aliquot of each hybrid was subjected to electrophoresis to confirm its purity.

Identifcation of the Site of Carboxymethylation - Each carboxymethylated Hb hybrid that was purified on CM-52 as described above was again separated into its α- and β-chains. The separated chains were treated with ethylenimine or with bromoethylamine and then with L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (11). The tryptic peptides were separated on an HPLC unit. The labeled peptides were detected by the presence of the ^{14}C-label after counting 500 µl of each 1 ml fraction. The radioactive fractions were pooled, evaporated to dryness in a Savant Concentrator, and hydrolyzed in 1 ml of 6 N HCl *in vacuo* for 22 hr. Amino acid analysis was performed on a Dionex D-500 amino acid analyzer for identification of the sites of modification by glyoxylate.

Determination of the Oxygen Equilibrium Curves of Carboxymethylated Hb - The Hb derivatives that were purified on CM-52 were concentrated to about 0.1

mM by ultrafiltration with an Amicon YM-10 membrane and then dialyzed against 0.05 M Bistris, 0.05 M Tris, pH 7.3. Prior to determination of the oxygen equilibrium curves on an Amino Hem-O-Scan, each sample was converted from the CO form to the oxy form as described previously (15) and finally concentrated to 1 mM in a collodion bag apparatus (Schleicher and Schuell). Experiments in the presence of 2,3-DPG were performed on Hb samples that had been previously mixed with the organic phosphate or NaCl such that the concentration of Hb was in the range of 0.32 to 0.48 mM and the DPG/Hb ratio was varied from 1-4. Experiments were also performed with the specifically carboxymethylated hybrids in the presence of varying NaCl concentrations (0.025 to 1 M). The P_{50} values were determined directly from the graphs of the Hem-O-Scan. For estimation of the Hill coefficient, the logarithmic values of the fractional saturation from 40-70% were plotted against the logarithmic value of the oxygen tension. The slope of the resulting straight line gave the n value.

Treatment of Hb with Glycolaldehyde and Estimation of the Amount of Cross-Linking - For these studies either unmodified Hb or carboxymethylated Hb (species Hb_1 or Hb_2 from the DE-52 column) was used. The concentration of Hb was about 0.02 mM in 50 mM KPO_4 buffer pH 7.3. Glycolaldehyde was added at a concentration of 50 mM unless otherwise indicated, and the cross-linking was performed at room temperature for varying periods of time, as described below. Samples for analysis were dialyzed extensively against 0.02 M sodium phosphate, pH 7.4.

In order to estimate the amount of cross-linking between tetramers (i.e., inter-tetrameric), a portion of the glycolaldehyde-treated Hb, dialyzed against 50 mM Tris acetate buffer, pH 7.3, was applied to a 2 x 110 cm column of Sephadex G-100. The column was eluted with 50 mM Tris acetate buffer pH 7.3, and the fractions were located by their absorption at either 280 nm or

420 nm. For estimation of the amount of subunit cross-linking, the Hb samples were analyzed by SDS gel electrophoresis on a 14% cross-linking acrylamide gel. The amounts of Hb loaded onto each gel were in the range of 5-10 μg. After staining the gel with Coomassie blue for 16 hrs and destaining in 30% methanol and 5% acetic acid, the amount of each cross-linked subunit was estimated by densitometry on a Gilford model 2520 instrument. The gel scans were quantitated by weighing of the area under each peak.

Amino Acid Analysis - For some samples amino acid analysis was performed in order to estimate either the degree of carboxymethylation or the extent of cross-linking as reflected by the decrease in lysine content. For such purposes the samples were dialyzed extensively against water prior to hydrolysis in 6 N HCl for 20-24 hrs. Estimation of the amounts of amino acid present was performed either on the amino acid analyzer.

Measurement of the Bohr Effects - The Bohr effects of $\alpha_2\beta_2$, $\alpha_2{}^{Cm}\beta_2$, $\alpha_2\beta_2{}^{Cm}$, and $\alpha_2{}^{Cm}\beta_2{}^{Cm}$ were determined by two methods. The proton titration technique described by other investigators (16) was used and the pH dependence of the oxygen affinity was also employed (7). The concentration of Hb was 60 μM in tetramer.

Analyses for chloride were performed on a dialyzed Hb solution before and after titrations. Before the titration, 0.2-0.6 mM chloride was found to be intrinsically bound to Hb. A Radiometer electrode was found to leak 5 mM chloride after immersion for 2 hrs in the Hb solution. A microelectrode (Microelectrodes Incorporated) leaked 1 mM chloride during the same time. The concentration of methemoglobin, which was checked repeatedly by the cyanmet procedure, was less than 10% for the data reported.

Other Methods - The procedures used for the competitive binding experiments and for the X-ray diffraction studies have been described in

detail in one of the manuscripts enclosed (Fantl et al, J. Biol. Chem., in press).

RESULTS

Sites of Carboxymethylation of HbA - In order to determine the sites of carboxymethylation of Hb, a 500 µl incubation mixture was dialyzed extensively against H_2O and the mixtures were subjected to acid hydrolysis in 6 N HCl for 16 hrs at 120°C. Each sample was then applied to a Dowex-2 column (0.9 x 22 cm) in order to quantitate the amounts of monocarboxymethyllysine, dicarboxymethylvaline, and monocarboxymethyllysine, as described in the Methods Section. The positions of the radioactive components that eluted from the Dowex-2 column were compared with the positions of the synthesized standards. At least 80% of the ^{14}C radioactive label was present in monocarboxymethylvaline under both oxy and deoxy conditions: The remaining 20% was found as ε-monocarboxymethyllysine. No dicarboxymethylvaline was detected under these conditions. In these studies the extent of modification of Hb by the incorporation of ^{14}C-glyoxylate was less than 2% of the total α-amino groups. Therefore, this distribution of carboxymethylation would reflect that attained under conditions of a kinetic steady state.

In order to locate the exact site of carboxymethylation on Hb, the β-chain was prepared from $\alpha_2\beta_2^{Cm}$ that had been purified by chromatography on CM-52. It was subjected to tryptic digestion after aminoethylation. The tryptic peptides were applied to an HPLC column as described in the legend to Fig. 2. The peptide map obtained from the β-chain of $\alpha_2\beta_2^{Cm}$ was identical with that of the β-chain of unmodified Hb. A single peak eluting at the position of βT-1 was found to contain all of the radioactivity. Amino acid analysis of this peptide confirmed its identity as βT-1 except for the loss of 0.7 residue of

valine, most likely due to carboxymethylation at this site. There was no detectable ε-^{14}C-carboxymethyllysine in the hydrolyzate, and lysine was recovered quantitatively (1).

Analysis of the α-chain of $\alpha_2{}^{Cm}\beta_2$ by HPLC revealed that all of the radioactivity was located in two peaks. The amino acid composition of the peak eluting at 26 min indicated that it was peptide αT-1. In order to purify the second radioactive peak, it was re-chromatographed with a more shallow gradient, and a single labeled peptide was obtained (1). Amino acid analysis of this peptide after acid hydrolysis indicated that its identity was αT-1+2. This peptide arises from incomplete tryptic cleavage at Lys-7 of the α-chain This linkage is known to be relatively resistant to trypsin even in the unmodified α-chain. Although each of the labeled peptides contains a potentially reactive amino group both at its COOH-terminal and at its NH_2-terminus, it is unlikely that the ε-NH_2 group of COOH-terminal lysine is carboxymethylated since tryptic cleavage would have been rendered impossible, and a completely different peptide map would have resulted. Since the peptide map on HPLC was virtually identical with the analysis of the unmodified chains, we concluded that the carboxymethyl group is on the NH_2-terminal residues. In support of this conclusion, no ε-carboxymethyllysine was found in αT-1+2.

Hb_3 was found to contain carboxymethyl groups at the N-terminal residues of the α- and β-chains by the HPLC peptide map procedures after tryptic digestion as described above. In addition, however, the tryptic digest of this species was found to contain carboxymethyl groups on other peptides and these are most likely due to reaction at lysine residues. Therfore, Hb_3 was considered an undesirable product and conditions were sought to minimize its formation.

Effects of the State of Ligation of Hb on the Profile of its Carboxymethylation - The distribution of N-carboxymethylated species described above were for Hb carboxymethylated under aerobic conditions. Comparison of those results with the chromatographic profile of the products formed furing the carboxymethylation of deoxy Hb (Fig. 2) indicates that although the position of each derivative is practically identical, their distribution is different. In addition, the overall extent of carboxymethylation is significantly increased when the starting material is deoxy Hb as indicated by the decreased amount of unmodified Hb (Hb_0) compared to that found when oxy Hb was carboxymethylated as described above. In addition, there is less of the undesirable Hb_3, when unliganded Hb is carboxymethylated. With deoxy Hb as the starting material, the amounts of desirable Hb_1 and Hb_2 are increased to nearly 75% of the total compared with about 50% of the total when oxy Hb is carboxymethylated.

Alkaline Bohr Effect - The measurements of the alkaline Bohr coefficients by the *proton titration method* is shown for $\alpha_2\beta_2$, $\alpha_2{}^{Cm}\beta_2$, $\alpha_2{}^{Cm}\beta_2{}^{Cm}$ in Fig. 4. The proton release at the maximum is shown in Table 2. For $\alpha_2\beta_2$ and $\alpha_2{}^{Cm}\beta_2$ these coefficients parallel each other over a wide range of chloride concentrations. From these data, we conclude that carboxymethylation of Val-1(α) results in a secondary amine with pK_a values in the deoxy and oxy states that are similar to those of the corresponding primary amine in unmodified Hb. Thus, in the presence of 0.1 M chloride it appears that chloride can bind effectively to Val-1(α) in deoxy $\alpha_2{}^{Cm}\beta_2$. However, the oxygen-linked binding of chloride may be slightly weaker in $\alpha_2{}^{Cm}\beta_2$ than in $\alpha_2\beta_2$, since the difference in the alkaline Bohr effect between 100 mM chloride and ~1 mM chloride is 0.40 protons/tetramer in $\alpha_2{}^{Cm}\beta_2$ versus 0.56 protons/tetramer in $\alpha_2\beta_2$.

In 0.5 M chloride, the alkaline Bohr coefficients of both $\alpha_2\beta_2$ and $\alpha_2{}^{Cm}\beta_2$ decrease slightly (by about 0.1 protons/tetramer) compared to their values at 0.1 M chloride. This result may be due to the oxygen-linked binding of chloride to sites other than Val-1(α). Another more likely possibility is that at 0.5 M chloride significant amounts of oxy Hb dimers are formed.

The alkaline Bohr coefficients of $\alpha_2\beta_2$ and $\alpha_2{}^{Cm}\beta_2{}^{Cm}$ also parallel each other over the chloride concentration range ~1 mM to 100 mM (see Table 2), again showing that carboxymethylation of Val-1(α) does not greatly alter the alkaline Bohr effect. Varying the chloride concentration from 100 mM to ~1 mM decreases the alkaline Bohr effect of $\alpha_2\beta_2{}^{Cm}$ by 0.48 protons/tetramer and of $\alpha_2{}^{Cm}\beta_2{}^{Cm}$ by 0.40 protons/tetramer, which is nearly the same as the corresponding decreases for the $\alpha_2\beta_2/\alpha_2{}^{Cm}\beta_2$ pair. This again implies that chloride binding to Val-1α) is only slightly decreased by carboxymethylation.

Comparing the alkaline Bohr coefficients of $\alpha_2\beta_2$ and $\alpha_2\beta_2{}^{Cm}$ as well as the coefficients of $\alpha_2{}^{Cm}\beta_2$ and $\alpha_2{}^{Cm}\beta_2{}^{Cm}$ (at constant chloride concentration) reveals that carboxymethylation of the β-chains decreases the alkaline Bohr effect by 0.50 protons/tetramer at ~1 mM chloride and by 0.54 protons/tetramer at 100 mM chloride. Since previous studies have shown the Val-1(β) does not contribute to the alkaline Bohr effect, carboxymethylation of Val-1(β) may create a secondary amine that has an increased pK_a in oxy Hb relative to its pK_a in deoxy Hb. Alternatively, the carboxymethyl group may preferentially interact with the basic residues of the 2,3-DPG binding site in oxy Hb to increase their pK_a values.

The alkaline Bohr coefficient was also calculated by the method of the pH dependence of the oxygen affinity. These results are described below. In general, the values obtained by this method correlate very well with those

found by the proton titration method on those samples in which both techniques were employed.

The Effect of Carboxymethylation on the Functional Properties of Hb - Earlier results (1) had shown that the unmodified and modified hybrid tetramer that had been subjected to the procedures of chromatography, chain separation, reconstitution, and re-chromatography retained the properties of the native molecule with respect to cooperativity (average Hill coefficient for the four hybrids = 2.4). This result imparts confidence that the manipulations and procedures used in this study do not adversely affect the properties of the protein and that the hybrid tetramer reflects the properties of native Hb.

The hybrid derivative $\alpha_2{}^{Cm}\beta_2$ has an intrinsic oxygen affinity (P_{50} = 12 mm Hg) that is considerably lower than that of the native protein (P_{50} = 7 mm Hg) (Fig. 4). This result can be explained by the propensity of small anionic moieties for the region around Val-1(α). The introduction of the negatively charged carboxymethyl moiety at this site creates a high local density of negative charge and minics the effect of very high concentrations of chloride in lowering the oxygen affinity of Hb. Addition of 2,3-DPG to the hybrid derivative $\alpha_2{}^{Cm}\beta_2$ lowers its oxygen affinity about 4-fold (to 48 mm Hb), which is about the same degree to which addition of 2,3-DPG lowers the oxygen affinity of unmodified Hb (to 33 mm Hg) (1). The equivalence point in the titration of unmodified $\alpha_2\beta_2$ and $\alpha_2{}^{Cm}\beta_2$ with 2,3-DPG is slightly greater than 1, most likely because these experiments were done in the absence of 0.1 N NaCl so there may be some nonspecific binding of 2,3-DPG. The effect of chloride on the hybrid $\alpha_2{}^{Cm}\beta_2$ also shows a lowering of oxygen affinity in response to the increasing anion concentrations (1). The difference between the initial and the final log P_{50} values is 0.5 for the unmodified hybrid and

respect to their ability to bind chloride.

The hybrid derivative $\alpha_2\beta_2^{Cm}$ has an oxygen affinity (P_{50} = 17 mM Hg) that is lower than either that of the unmodified tetramer or of $\alpha_2^{Cm}\beta_2$ (1). This result is consistent with known avidity for anions in the region comprising the cleft between the two β-chains and the consequent lowering of the oxygen affinity of Hb upon binding of such anions. However, the covalent attachment of the carboxymethyl group in this cleft prevents a further lowering of the oxygen affinity by 2,3-DPG to any significant degree even at high DPG-Hb rations (maximum P_{50} = 25 mm Hg). In this respect, the covalently bound carboxymethyl group mimics the effect of very high concentrations (0.5 M) of chloride in obviating the effect of 2,3-DPG. In contrast, the addition of chloride to $\alpha_2\beta_2^{Cm}$ results in a significant lowering of the oxygen affinity (1). This effect is most likely due to the binding of the chloride anion to the region around the NH_2-terminus of the α-chain, which is free in $\alpha_2\beta_2^{Cm}$.

The intrinsic oxygen affinity of the tetramer that is fully carboxymethylated at its NH_2-terminal residues, $\alpha_2^{Cm}\beta_2^{Cm}$ (P_{50} = 37 mm Hg), is much lower than the additive effects of such modification at the individual chains (Fig. 4). The addition of 2,3-DPG to this hybrid results in a further lowering of the oxygen affinity (P_{50} = 50 mm Hg), although the magnitude of this effect is far less than that observed for unmodified Hb in the presence of 2,3-DPG (1). Since the hybrid $\alpha_2^{Cm}\beta_2^{Cm}$ already has two of the anion-binding regions replaced covalently with a negatively charged anion, further lowering of the oxygen affinity by chloride might be marginal.

For all of the hybrids studied (except for $\alpha_2^{Cm}\beta_2^{Cm}$ at low oxygen tensions), the degree of cooperativity was high (Fig. 5, inset). This finding argues against significant distortion of subunit contacts after introduction

of the negatively charged carboxymethyl group. As indicated above, we have also examined the pH dependence of the oxygen affinity in a few studies.

The pH dependence of the oxygen affinity for unmodified $\alpha_2\beta_2$ was compared with that of $\alpha_2^{Cm}\beta_2^{Cm}$ (Fig. 5). On the left panel is shown the oxygen affinity, expressed as log P_{50} as a function of pH, for $\alpha_2\beta_2$. The slope of this line, which is a measure of the Bohr coefficient, has a value of about -0.51 close to the reported values (7). The data in the right panel of Fig. 5 show that the line generated as a plot of log P_{50} versus pH is elevated. This fact indicates that the individual oxygen equilibrium curves of $\alpha_2^{Cm}\beta_2^{Cm}$ at each pH have shifted significantly to the right. The Bohr coefficient calculated from the slope of this line is lowered to a value of -0.36. These values are nearly identical to those measured for the same derivatives at pH 7.2 by the proton titration method described above. The structural reasons for the lowering of the Bohr effect after carboxymethylation of the terminal amino groups may be due to the carboxymethyl group on the termini of the β-chain protruding fairly deep into the DPG cleft as assessed by X-ray diffraction studies as described below.

Cross-Linking of Unmodified Hb - The inital studies on the cross-linking of Hb with glycolaldehyde were performed with unmodified HbA in the liganded state. At a constant glycolaldehyde concentration of 50 mM and a Hb concentration of 0.02 mM at 25°C, aliquots were removed at several intervals during a 24 hr period. The extent of reaction between Hb subunits was determined by SDS polyacrylamide gel electrophoresis, as shown in Fig. 6. It is evident that the cross-linking of Hb subunits by glycolaldehyde is a relatively slow process. Thus, after treatment with glycolaldehyde for 1, 3, and 5 hrs there was a gradual increase in the amount of two cross-linked subunits of 32,000 molecular weight, and a corresponding decrease in single Hb

subunits (Fig. 6, lanes f-h). For the samples incubated from 1-5 hrs, there was no detectable loss in lysine content most likely because of the large amount of unmodified, monomeric subunits present. For the sample taken at 24 hrs (lane i) there was a large amount of very high molecular weight aggregate and a concomitant disappearance of single Hb subunits. Under these conditions, the lysine content was decreased by up to 30% with no detectable effect upon the other amino acids.

In order to provide an indication of the extent of cross-linking between Hb tetramers, gel filtration of native cross-linked Hb was performed on Sephadex G-100 (Fig. 7). After 3.5 hrs of incubation of glycolaldehyde with a sample of carboxymethylated Hb, gel filtration showed a large peak of Hb which eluted in the area of 64,000 molecular weight. In addition, a significant amount of higher molecular weight species, which represented about 15% of the total material, eluted near the void volume of the column. These latter species most likely represent cross-linked Hb tetramers of 128,000 molecular weight and larger, as judged by the slight inflections in the curve. SDS gel electrophoresis of the 64,000 molecular weight material indicated the presence of 19% of two cross-linked subunits with the remainder of the material as uncross-linked subunits (data not shown). A similar analysis for the higher molecular species showed a 13% of three cross-linked subunits, 29% of two cross-linked subunits, and the remainder as uncross-linked subunits.

Oxygen Equilibrium Curves of Carboxymethylated and Cross-Linked HbA - Treatment of unmodified Hb with glycolaldehyde for intermediate periods of time (5 hrs) results in the formation of both intra- and inter-tetrameric cross-links corresponding to 22% of two cross-linked subunits and 4% of three cross-linked subunits in an unfractionated sample as described above. In order to determine whether such treatment had an adverse effect on the

molecule, i.e., formation of met-Hb or partial denaturation, the oxygen equilibrium curve of this population of Hb molecules was determined. The oxygen affinity of this sample was slightly higher (P_{50} = 7 mm Hg) than that of unmodified Hb (P_{50} = 9 mm Hg, Fig. 18, curve A) and the Hill coefficient was lowered from 2.5 to an average n value of 1.6. Thus, the cross-linking of Hb by glycolaldehyde leads to a slight increase in its oxygen affinity and a decrease in its cooperativity, which is anticipated if some subunits were not free to function independently because of the cross-linking.

The oxygen equilibrium curve of $\alpha_2^{Cm}\beta_2^{Cm}$ (Fig. 8, line C) shows that its oxygen affinity is lowered even further at 37°C (to a value of 30 mm Hg) than it is at 25°C (Fig. 4). The Hill coefficient for this sample is 2.8, which indicates, that Hb fully carboxymethylated at its N-terminal amino groups has not undergone a drastic alteration in its structure.

Hb carboxymethylated on its four N-terminal residues and then cross-linked with glycolaldehyde had an oxygen equilibrium curve with an average P_{50} of 14 mm Hg and a Hill coefficient of 2.0 (Fig. 9, line B). Thus, carboxymethylated, cross-linked Hb releases its oxygen more readily than either unmodified Hb or cross-linked, unmodified Hb.

DISCUSSION

Means and Feeney (17) demonstrated that the procedure of reductive alkylation is selective for the amino groups of protins with no alkylation of cysteine, histidine, or methionine residues. The lack of reactivity of these latter amino acid residue resdieus is due to the fact that they do not form Schiff bases adducts with the aldehyde that could be subsequently reduced by sodium cyanoborohydride. Carboxymethylation of lysine residues of antigen E from ragweed pollen was achieved by King et al (18) by reductive alkylation

with glyoxylate and $NaCNBH_3$. In our studies, selectivity for the NH_2-terminal residues of each chain of Hb was achieved by reductive alkylation at neutral pH rather than at the alkaline pH that is usually employed for reductive alkylation. By manipulation of conditions such as pH and ratio of reactants to Hb we have selectively (but not exclusively) modified the N-terminal amino groups of the α- and β-chains of Hb. This has been demonstrated by peptide mapping and subsequent amino acid analysis, and more recently, these conclusions have been corroborated by X-ray diffraction studies described below. Chromatographic separation of these derivatives was possible because of the extra negative charge conferred on them by the carboxymethyl group.

Except for the desirable property of a low oxygen affinity, carboxymethylated Hb retains many of the native properties of Hb including its cooperativity, thus indicating that the conditions whereby the carboxymethyl group is introduced on the N-terminal amino groups of Hb do not effect the inegrity of the molecule. The nature of this carboxymethylation reaction is quite different from the carboxymethylation of amino acid side chains with iodoacetate. This latter reaction is much less specific than reductive carboxymethylation since in addition to modification of amino groups, it can lead to extensive modification of other residues.

In recent years, it has become apparent that any loss of net positive charge in the DPG binding pocket, either by specific chemical modification or by a mutational event, serves to lower the intrinsic oxygen affinity of such a Hb molecule. The lower intrinsic oxygen affinity of $\alpha_2\beta_2^{Cm}$ (in the absence of DPG) is consistent with this hypothesis. In addition, the inability of DPG to lower the oxygen affinity of the $\alpha_2\beta_2^{Cm}$ derivative is consistent with observations with other Hbs, such as acetylated fetal Hb in which the net positive charge of the DPG binding site has been reduced. However, the

availability of binding sites for chloride at other binding sites in the hybrid $\alpha_2\beta_2^{Cm}$ readily explains the lowering of the oxygen affinity in the presence of this anion.

These carboxymethylated derivatives (Hb-NH-CH_2-COO^-), which are easily prepared in their native state, should be useful in answering questions regarding the binding of anions or CO_2 to Hb (Hb-NH-COO^-) under a variety of experimental conditions. The possibility of mimicing the effects of the natural allosteric effector, CO_2 in reducing the oxygen affinity of Hb is suggested by the results of our collaboration with Dr. A. Arnone (described next).

Carboxymethylated Hb as an Analogue of Carbamino Hb - The X-ray studies (in press, copy enclosed) show that deoxy Hb modified by carboxymethylation of Val-1(α) and Val-1(β) is structurally very similar to the corresponding carbamino adducts of deoxy Hb. In both types of derivatives only minor shifts of the NH_2-terminal peptides are detected, and the corresponding terminal carboxyl groups form the same intrasubunit contacts. Specifically, for both the carbamino adduct (HbNHCOO^-) and the carboxymethyl derivative (HbNHCH_2COO^-) at the α-chain, the carboxyl group in each case forms an intrasubunit hydrogen bond with the side chain of Ser-131(α). Similarly, the carboxyl group of each species on the β-chain forms intrachain ionic contacts with Lys-82(β). The functional similarities of carboxymethylated Hb and carbamino Hb with respect to the decreased oxygen affinity are consistent with these structural similarities. Thus, carboxymethylation of both the α- and β-chains ($\alpha_2^{Cm}\beta_2^{Cm}$) reduces the oxygen affinity of Hb by a factor of 5 (1), which is comparable to the reduction in Hb oxygen affinity produced by high levels of CO_2 (19).

The Alkaline Bohr Effect - A number of studies have shown that a large fraction of the alkaline Bohr effect is due to an oxygen-linked pK_a shift to

the α-amino group of Val-1(α). As discussed below, the stereochemical mechanism for this pK_a shift is now thought to involve the binding of chloride to Val-1(α) in deoxy Hb (20, 21). The carboxymethyl moiety, covalently bound at a specific site on Hb, can be considered as a small covalently bound anion which approximates the size and charge of the chloride anion as well as its similarity to the CO_2-Hb adduct, the carbamate. The lowered oxygen affinity of carboxymethylated Hb is most likely due to the decreased positive charge within the DPG cavity as a result of the presence of the negatively-charged carboxymethyl group bound covalently. This phenomenon has been found for other chemically modified (22) and mutant Hbs (23) and a structural reason for it is currently under study. In addition, it is clear from inspection of its structure that the carboxymethyl adduct could easily be considered as an analogue of carbamino Hb, in which carbon dioxide is carried on the amino groups of Hb. However, in the latter case this derivative is not stable and carbon dioxide is rapidly released. On the other hand, the carboxymethyl derivative with a methylene group introduced into the carbamate structure is a stable covalent modification of the protein and permits study of the effects of anions bound to the protein.

The previous studies on the cross-linking of proteins with the mild agent glycolaldehyde, that we reported earlier for RNase (24), have now been successfully extended to the oligomeric protein, Hb. The question of the optimum molecular weight for cross-linked Hb (i.e., 64,000 or 128,000) is one that is under investigation. In the present study where the majority of the cross-linked tetramers are of 64,000 molecular weight, it is possible that there was a fair amount of αβ dimer present at the low initial Hb concentrations used. This factor may be subject to manipulation that could lead to the formation of a different profile of products. The relatively slow

rate of cross-linking may be an advantage since more control of the reaction would be possible than with a potent cross-linking agent such as glutaraldehyde. Cross-linking by glutaraldehyde produces very high molecular weight species even after a short exposure. Further studies on the conditions of cross-linking of carboxymethylated Hb to obtain predominatly either 64,000 or 128,000 or greater molecular weight species are in progress. With the procedures described in this report, it should be possible to obtain a selective enrichment of one species or the other for further testing as a potential blood substitute.

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Table 1

Alkaline Bohr Coefficients of Unmodified and Carboxymethylated Hemoglobin Hybrids

The alkaline Bohr effect of each sample was measured by the technique of proton titration as described in the Methods and the legend to Fig. 5. The results are expressed on a tetramer basis.

	Protons Released per Hb Tetramer			
Conditions	$\alpha_2\beta_2$	$\alpha_2{}^{Cm}\beta_2$	$\alpha_2\beta_2{}^{Cm}$	$\alpha_2{}^{Cm}\beta_2{}^{Cm}$
Low salt (0.2 mM to 0.6 mM intrinsically bound)	1.56	1.60	1.04	1.12
0.1 M Cl^-	2.12	2.00	1.52	1.52
0.5 M Cl^-	2.04	1.88	1.56	1.76

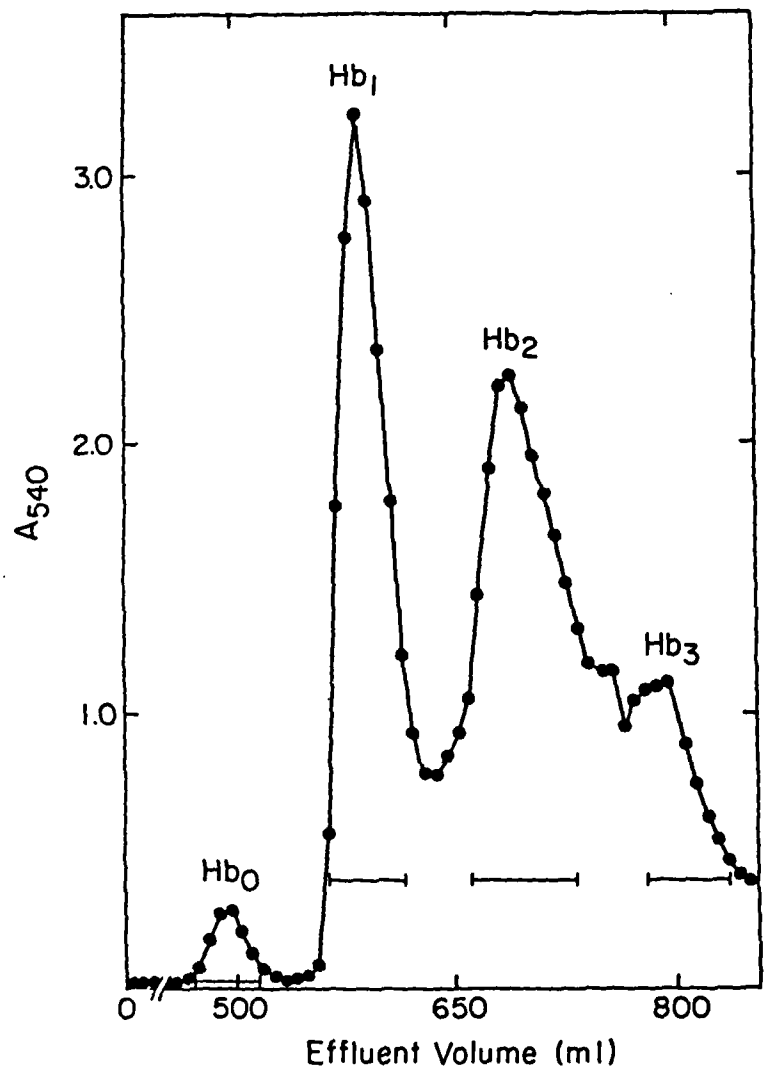

Fig. 1 - Separation of Carboxymethylated Derivatives of Hb on DE-52. The separation was achieved as described in the text. For analysis of the various species, the fractions designated by the arrows were pooled. Oxy HbA was carboxymethylated.

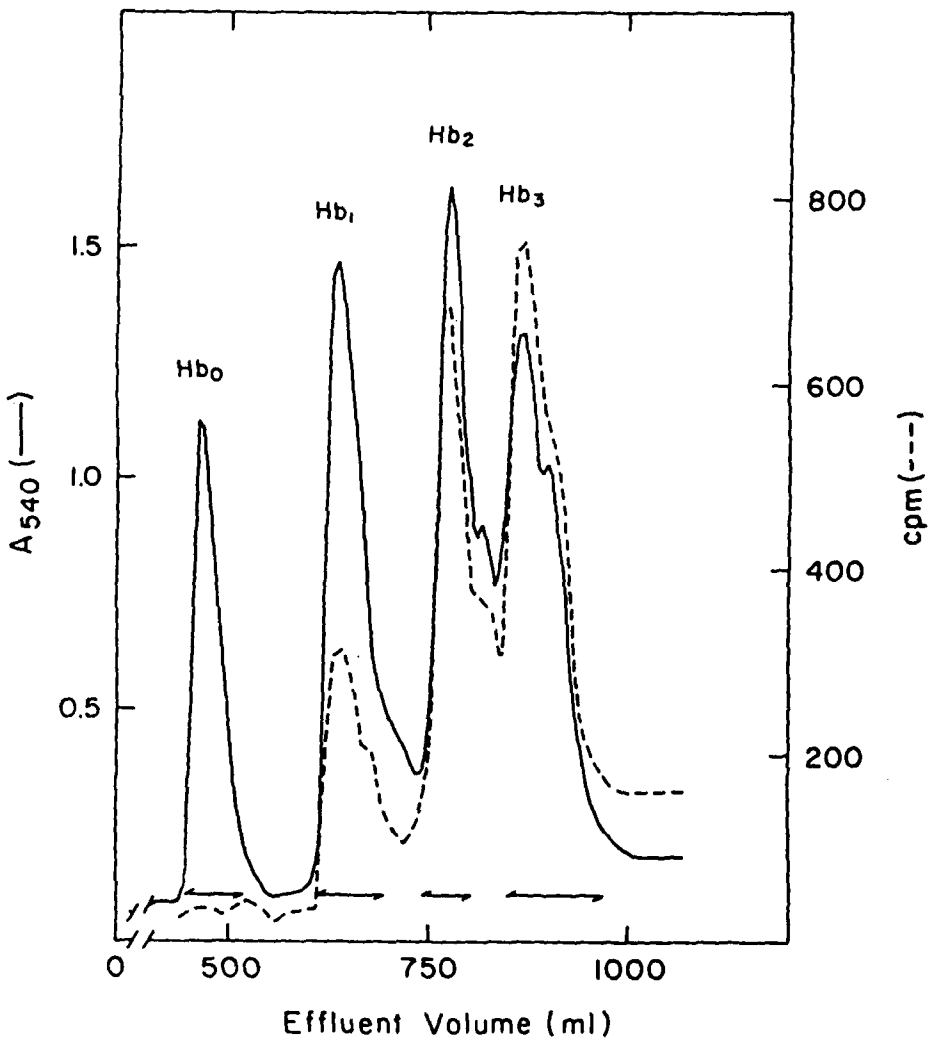

Fig. 2 - Separation of Carboxymethylated Derivatives of Hemoglobin on DE-52. The sample of deoxy hemoglobin A was carboxymethylated as described in the text. After dialysis the sample was applied to a column and eluted with the gradient as described in the text. The designations Hb_0, Hb_1, Hb_2, and Hb_3 are defined in the text.

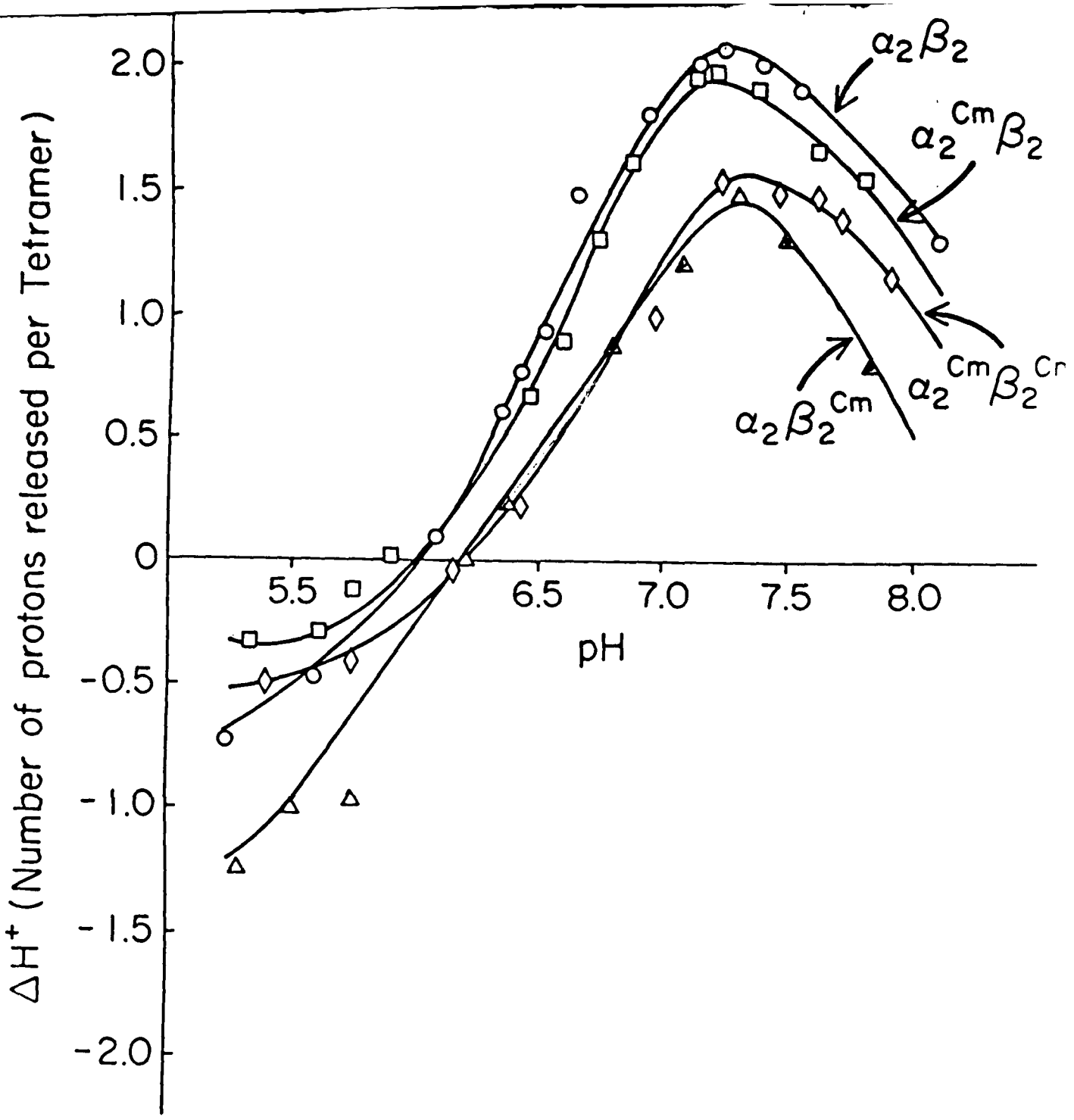

Fig. 3 - Bohr Effect Measured According to the Proton Release Technique as Described in the Methods. Samples at a concentration of 60 uM in tetramer were deoxygenated in the presence of 0.1 M chloride. After oxygenation, a standardized solution of 10 mM NaOH was added from an autoburrette to raise the pH of the oxy hemoglobin solution to the value measured in the deoxy form. Below pH 6, since the pH was greater in oxy than deoxy hemoglobin, the NaOH was added to the deoxy hemoglobin solution until the value was raised to the value of the pH registered by the oxy hemoglobin solution. Thus, ΔH^+ was calculated from the known aliquot of base added.

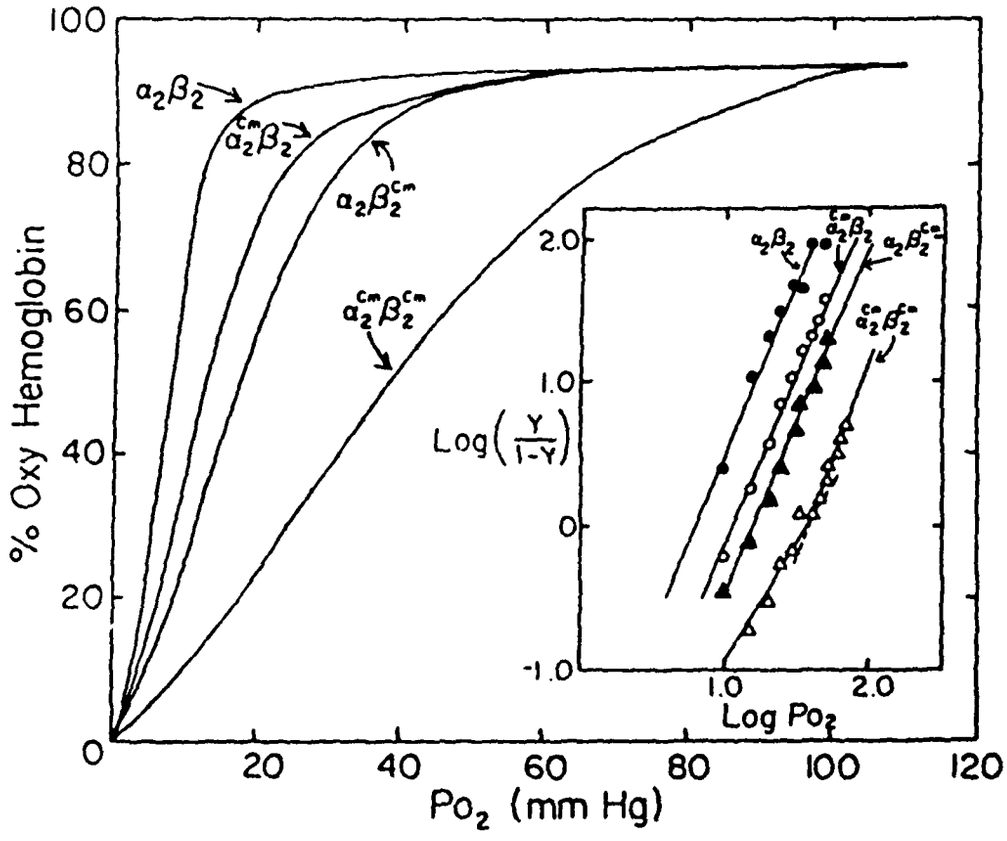

Fig. 4 - Oxygen Equilibrium Curves of Unmodified and Specifically Carboxymethylated Hybrids of HbA. The samples were prepared as described in the text, and 4 ul portions were used for determination of the oxygen equilibrium curve in a Hem-O-Scan instrument.

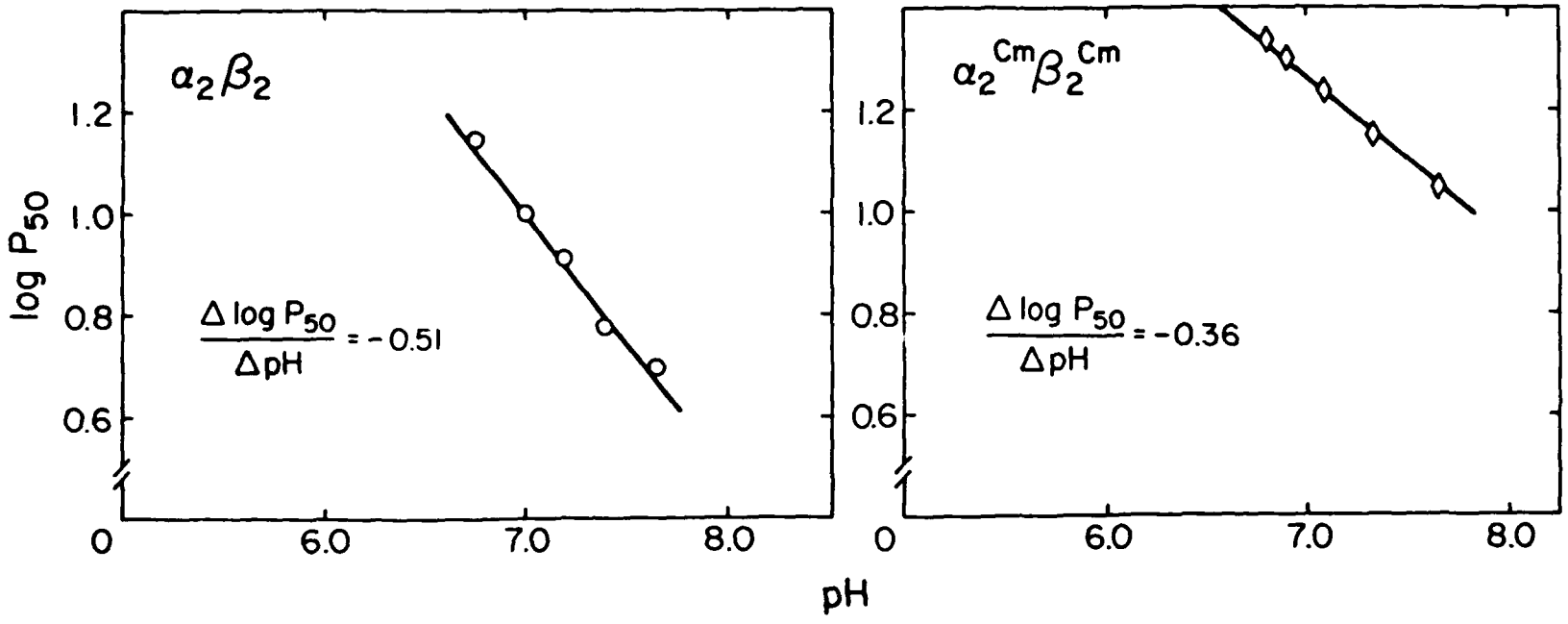

Fig. 5 - pH Dependence of the Oxygen Affinity of Unmodified and of Carboxymethylated Hemoglobin A. The oxygen affinity of the samples in the presence of 0.1 M chloride at each pH value was determined on an Aminco Hem-O-Scan instrument as described in the text. This experiment was carried out at 25°C.

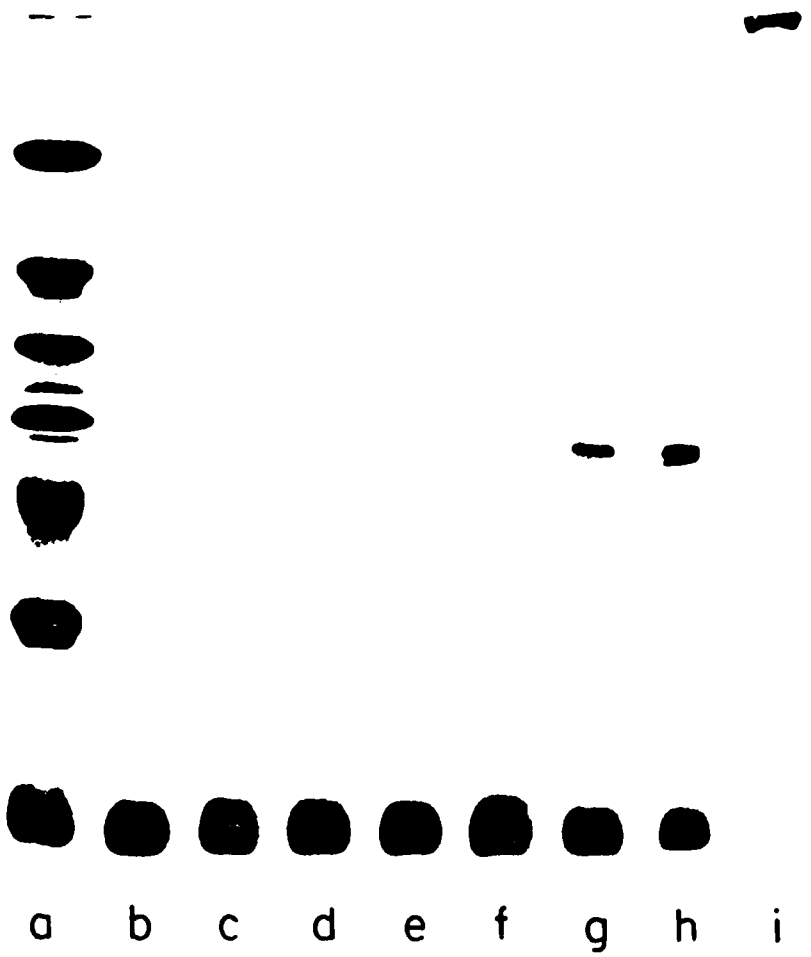

Fig. 6 - SDS Gel Electrophoresis of Hemoglobin Before and After Treatment with Glycolaldehyde. Lane a contains a standard mixture of proteins of defined molecular weight from 14,000 (bottom) to 66,000 (top). The fourth band from the bottom of the gel is carbonic anhydrase with a molecular weight of 29,000. The hemoglobin samples was incubated in buffer alone for 1 hr (lane b), 3 hrs (lane c), 5 hrs (lane d), or 24 hrs (lane e). Unmodified hemoglobin A was treated with glycolaldehyde as described in the text for 1 hr (lane f), 3 hrs (lane g), 5 hrs (lane h), or 24 hrs (lane i).

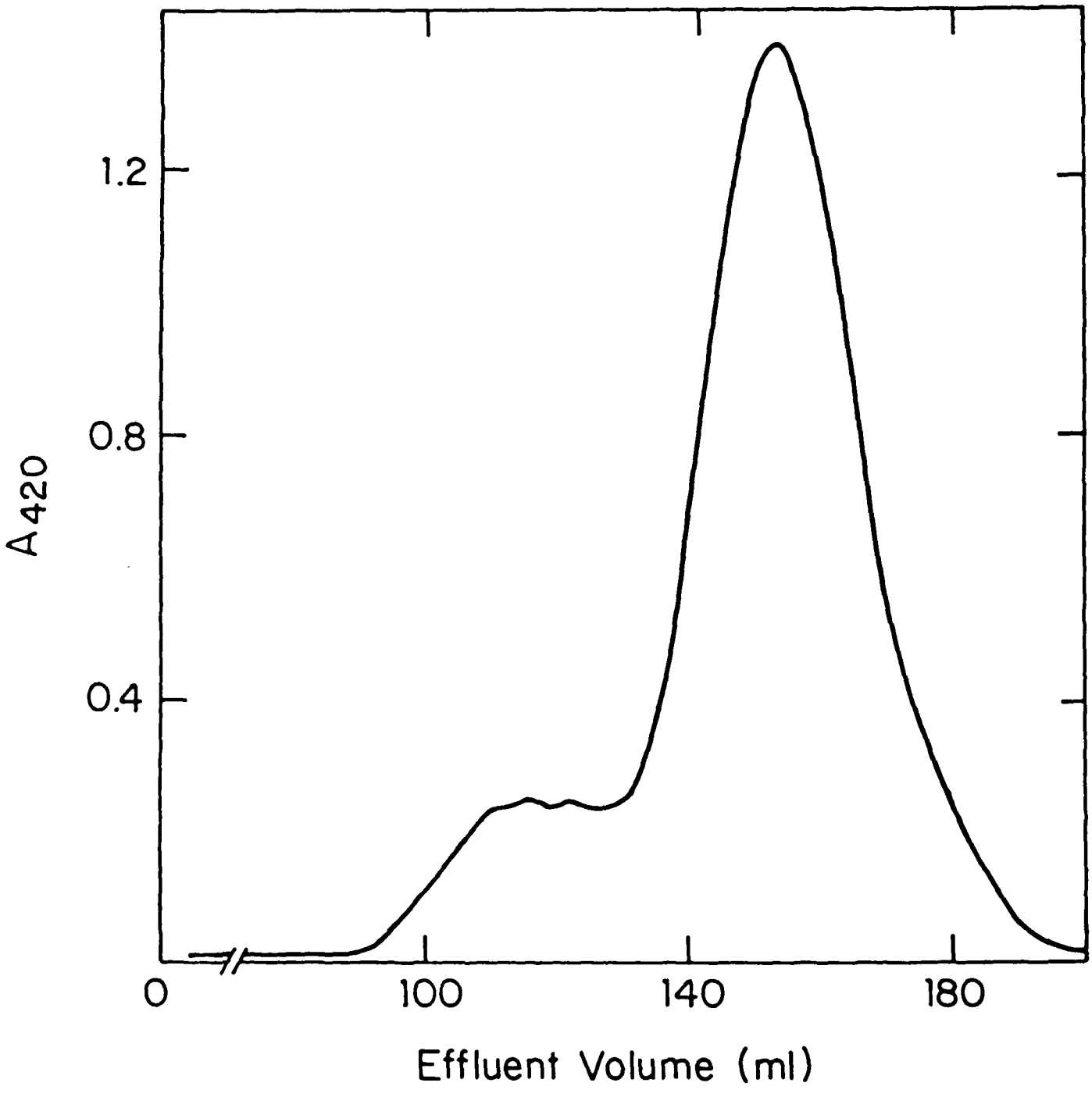

Fig. 7 - Gel Filtration of Cross-Linked Hemoglobin. A sample of carboxymethyalted hemoglobin was treated with 42 mM glycolaldehyde for 3.5 hrs. This sample was applied to a column of Sephadex G-100 and eluted as described in the text.

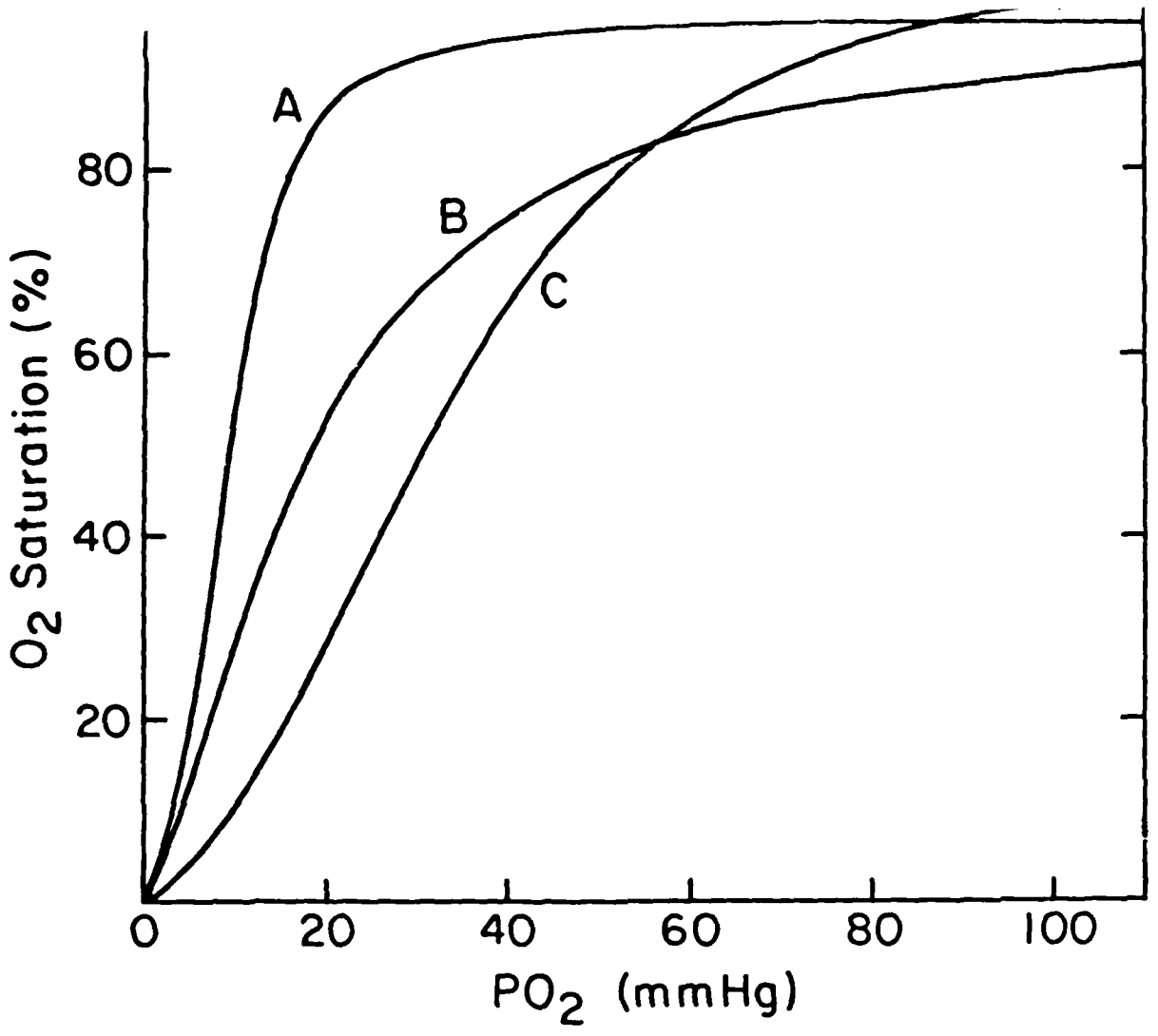

Fig. 8 - Effect of Cross-Linking and Carboxymethylation on the Oxygen Equilibrium Curve of Hemoglobin A. The oxygen equilibrium curves of these samples was determined in 50 mM bis-Tris, pH 7.5 and the experiments were carried out at 37°C as described in the text on an Aminco Hem-O-Scan instrument. Curve A - Unmodified hemoglobin A. Curve B - Carboxymethylated hemoglobin A (component Hb_2 of Fig. 1) was treated with 50 mM glycolaldehyde for 5 hrs as described in the text. Curve C - Carboxymethylated hemoglobin A corresponding to component Hb_2 of Fig. 1.


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